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The use of hydrazine based derivatization reagents for improved sensitivity and detection of carbonyl containing compounds using MALDI-MSI

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Abstract

Hydrazine based derivatization reagents have been used to detect the presence of the carbonyl containing glucocorticoid fluticasone proprionate in rat lung tissue by MALDI-MSI. Such reagents also act as a matrix for analysis by MALDI-MS and have been termed "reactive matrices". Cryosections of rat lung tissue (12 μm) spotted with a range of concentrations of fluticasone proprionate, were derivatized in situ with 2,4-dinitrophenylhydrazine (DNPH) and 4-Dimethylamino-6-(4-methoxy-1-naphthyl)-1, 3, 5-triazine-2-hydrazine (DMNTH) by the use of an acoustic reagent spotter. It has been demonstrated that DMNTH gave superior results compared to DNPH and that analysis of samples immediately after application of DMNTH resulted in the detection of the protonated hydrazone derivative ($[M_D+H]^+$) of fluticasone propionate at a concentration of 500 ng/μL. It has been further shown that a prolonged reaction time (~48 hours) improves the detection limit of the protonated hydrazone derivative to 50 ng/μL and that improvements in sensitivity and limits of detection are obtained when a conventional MALDI matrix CHCA is employed in conjunction with the DNPH/ DMNTH reactive matrix.

Keywords: Derivatization, MALDI-MSI, reactive matrices, glucocorticoids.

1. Introduction

Corticosteroids are the mainstay of asthma management and are used to treat the inflammatory response associated with the disease. Depending on the severity of asthma the amount of fluticasone propionate a patient must inhale can range from 150-1000 µg two times per day (www.medicines.org.uk). The ability to monitor their distribution in the lungs would be useful to assess the efficiency of drug delivery. Matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) provides a label free method to monitor the distribution of pharmaceutical compounds in biological tissue sections. As part of a project to study the distribution of corticosteroids in dosed rat lung tissue sections by MALDI-MSI, methodologies for on-tissue derivatization have been studied. Corticosteroids contain multiple carbonyl functionalities which are not easily protonated/ de-protonated under mass spectrometry conditions, due to their low ionisation efficiencies. Derivatization has been proposed as a solution to this problem by introduction of groups with fixed charges or with high proton affinity. An additional benefit of the use of derivatization in "soft" ionisation mass spectrometry techniques such as MALDI-MS is that it can help prevent matrix-related ion suppression and isobaric overlay between matrix and analyte peaks by the introduction of groups with fixed charges or easily ionisable groups [1].

The most common derivatization reactions of carbonyl compounds reported for analysis by "soft" ionisation mass spectrometry methods are based on the formation of oximes and hydrazones [1]. Other published methods involve the formation of Schiff's bases, semicarbazones and thiosemicarbazones [2]. Whilst there are many derivatization reagents available, there is not, in reality, a preferred universal reagent. Thus the choice is usually determined by experimentation. There are several requirements to consider when choosing the ideal derivatization reagent for use: 1) the reagent should be pre-charged or include a group having high proton affinity, 2) it must contain a suitable reactive group and 3) preferably it should be commercially available or synthesised using inexpensive reagents [1, 3]. Careful consideration should be made when selecting the derivatization reagent for example smaller reagents are suited for sterically hindered functional groups, the

addition of a larger reagent can have the desired effect of moving the mass of the derivative into the higher mass region of the mass spectrum [2].

2,4-Dinitrophenylhydrazine (DNPH) is the most commonly used hydrazine based reagent for derivatizing the carbonyl functionalities of aldehydes and ketones for liquid chromatographic separation followed by UV/Vis or fluorescence detection [4]. DNPH is characterised as a "reactive matrix" as it not only derivatizes the carbonyl containing compounds but also assists in desorption and ionisation in MALDI-MS [4]. DNPH has been used previously for the derivatization and analysis of corticosteroids by MALDI-MS [5]. The analysis of the derivatized corticosteroids showed a radical cation ($[M]^{+\bullet}$) which is formed via charge transfer reactions, as well as the protonated derivatized molecule ($[M_D+H]^+$) [5]. This effect has been studied here.

DNPH has also been used for the derivatization of phospholipids and their related oxidation products for analysis by MALDI-MS [6]. DNPH or a mixture of DNPH and CHCA has been utilised for the direct analysis of formalin-fixed paraffin embedded (FFPE) tissue samples, that had been stored for less than 1 year. DNPH was used to neutralise any residual formalin molecules in order to improve the signal of peptides greater than 5000 kDa and adducts corresponding to (protein-N=CH2) were suppressed [7]. Recently it has been shown that digestion of spermatozoa samples with phospholipase A_2 followed by derivatization with DNPH, can be used for the identification of plasmalogens in complex lipid mixtures [8].

4-Dimethylamino-6-(4-methoxy-1-naphthyl)-1, 3, 5-triazine-2-hydrazine (DMNTH) is a “tailor-made” derivatization reagent that combines selective reactive functionality, stability and good spectroscopic properties in one compound [9]. This reagent was developed by the Karst group at the University of Münster, Germany. It is synthesised by substitution reactions of cyanuric chloride, which acts as a base molecule to couple functional groups that have specific roles in the detection, reactivity and polarity of the derivatization agent. There are several reports on the use of DMNTH as a derivatization reagent. Examples are the derivatization of carbonyl containing compounds followed by chromatographic separation and detection by atmospheric pressure chemical ionisation (APCI) mass spectrometry and in water samples [10, 11]. The optimised parameters necessary for the separation of DMNTH derivatized carbonyl compounds by reversed phase capillary electrochromatography (CEC) have been described [12].

Test tubes coated with DMNTH have been used for the determination of aldehydes in air samples followed by chromatographic separation and detection by UV-Vis and fluorescence spectroscopy, the results were found to be comparable to the established method using the derivatization reagent DNPH [13]. The most recent application of DMNTH reported [9], is for the derivatization and analysis of a range of carbonyl containing compounds by laser desorption ionisation-mass spectrometry (LDI-MS).

The hydrazine based reagents DNPH and DMNTH have been employed for the derivatization of a range of glucocorticoids both in-solution and on-tissue to form their respective hydrazones and improve their mass spectral ionisation efficiency and detection. In the work reported here fluticasone propionate has been selected to demonstrate the utility of the methodology as widely used glucocorticoid containing a carbonyl functionality.

2. Methods

2.1. Information

All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals.

2.2. Materials

The reagents α-cyano-4-hydroxycinnamic acid (CHCA), fluticasone propionate ($C_{25}H_{31}F_3O_5S$, MW 500.57), beclomethasone dipropionate ($C_{28}H_{37}ClO_7$, MW 520.22), budesonide ($C_{25}H_{34}O_6$, MW 430.23), trifluoroacetic acid (TFA) and ALUGRAM1 SIL G/UV254 precoated aluminum sheets were purchased from Sigma Aldrich® (Gillingham, Dorset, UK). The solvents methanol (MeOH), ethanol (EtOH) and acetonitrile (ACN) were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Dexamethasone ($C_{22}H_{29}FO_5$, MW 392.19) was purchased from TCI Europe (Belgium). 2, 4-dinitrophenylhydrazine (DNPH) was purchased from Alfa Aesar® (Heysham, Lancashire, UK). 4-Dimethylamino-6-(4-methoxy-1-naphthyl)-1, 3, 5-triazine-2-hydrazine (DMNTH) was kindly donated by Professor Uwe Karst (University of Münster, Germany).

2.3. Instrumentation

Mass spectra and images were acquired in positive ion mode on an Applied Biosystems/MDS Sciex hybrid quadrupole time-of-flight mass spectrometer (Q-Star Pulsar-i) with an orthogonal MALDI ion source (Applied Biosystems, Foster City, California, USA) and a high repetition Neodymium-doped yttrium vanadate (Nd: YVO_4) laser (355nm, 5 KHz) (Elforlight Ltd, Daventry, Northamptonshire, UK). Image acquisition was performed at a spatial resolution of 150 µm × 150 µm in "Raster Image" mode; images were generated using the freely available Novartis Biomap 3.7.5.5 software (www.maldi-msi.org).

2.4. Tissue preparation

Frozen control rat lung tissue was sectioned using a Leica cryostat (Leica Microsystems, Wetzlar, Germany) to produce 12 µm thick sections, which were thaw mounted onto clean aluminium sheets.

2.5. Derivatization of glucocorticoids using DNPH

2.5.1. In-solution derivatization procedure

The derivatization procedure involved the incubation of 10 µL of a test steroid (0.5 mg/mL in acetonitrile) and 10 µL of the solution of the DNPH matrix (1 mg/mL in acetonitrile with 0.3 % hydrochloric acid) in a 0.5 mL micro-centrifuge tube at room temperature for 30 minutes.

The derivatized steroids were then analysed using the Q-Star instrument operated in positive ion mode with an accumulation time of 0.73 seconds, declustering potential of 0, focusing potential of 20, second declustering potential of 15, collision gas (nitrogen) of 3 arbitrary units (the three potentials and collision gas parameters are the same for each experiment and will not be quoted again from this point) and the scanning duration of 1 minute with a mass range of m/z 150-1000.

2.5.2. On-tissue derivatization procedure

Fluticasone proprionate standards (500, 100, 50, 25 and 5 ng/µL prepared in 70 % acetonitrile) were spotted (1 µL) onto a control rat lung tissue section in triplicate. Then 1 µL of a 4 mg/mL solution of DNPH in 50: 50 acetonitrile: water (v/v) with 0.1 % TFA was applied onto the spotted areas (10 times per spot and allowed to dry between applications).

Using the Labcyte Portrait 630^{TM} reagent multispotter (Labcyte, California, USA) an acoustic reagent spotter which is described elsewhere [14], the reactive matrix (4 mg/mL solution of DNPH in 50: 50 acetonitrile: water (v/v) with 0.1 % TFA) was deposited onto the spotted lung tissue (20 cycles with 200 μm distance between spots).

Imaging experiments were performed using the Q-Star instrument with the "Raster Image" mode set to the "slow" speed, the instrument was operated in positive ion mode with an accumulation time of 0.29 seconds, ion release delay of 67.1 (the width of the pulse of ions released by the collision cell into the TOF), ion release width of 29.1, duration of scanning of 2 minutes and a mass range of m/z 150-700 with enhancement of m/z 500.2.

2.6. Derivatization of glucocorticoids using DMNTH

2.6.1. In-solution derivatization procedure

The derivatization procedure involved the incubation of 10 μL of a test steroid (2 mg/mL in acetonitrile) and 10 μL of the DMNTH matrix (5 mg/mL in 50: 50 acetonitrile: water (v/v) with 0.1 % TFA) in a 0.5 mL micro-centrifuge tube at room temperature for 48 hours.

The derivatized steroids were analysed using the Q-Star instrument operated in positive ion mode with an accumulation time of 0.73 seconds, the scanning duration of 1 minute with a mass range of m/z 250-900.

2.6.2. On-tissue derivatization procedure

Fluticasone proprionate standards (2000, 1000, 500, 250, 100, 50, 20 and 10 ng/μL prepared in 70 % acetonitrile) were spotted (1 μL) onto a control rat lung tissue section. Using the Labcyte Portrait 630^{TM} reagent multispotter, the reactive matrix (5 mg/mL solution of DMNTH in 50: 50 acetonitrile: water (v/v) with 0.1 % TFA) was deposited onto the spotted lung tissue (25 cycles with 200 μm spot-to-spot distance).

Once the spotted tissue section had been coated with the reactive matrix, it was placed onto a polystyrene block and placed into a glass chamber containing 50 % methanol. The chamber lid was sealed using parafilm and incubated at 37°C with 5 % carbon dioxide for 48 hours in a Heraeus, Heracell incubator in order to create a humid environment.

Following incubation the section was analysed using the Q-Star instrument with the “Raster Image” mode set to the "slow" speed, the instrument was operated in positive ion mode with an accumulation time of 0.49 seconds, ion release delay of 84.5, ion release width of 36.6, duration of scanning was 3 minutes and a mass range of m/z 450-800 with enhancement of m/z 793.2.

2.7. Data processing

Mass spectra from Analyst were exported in the form of text files and imported into mMass, an open source mass spectrometry software used for mass spectral processing [15].

3. Results and Discussion

3.1. MALDI-MS analysis of glucocorticoids

To illustrate the challenge of analysing glucocorticoids with MALDI-MS, a solution (500 ng/µL) of fluticasone propionate was mixed with the matrix CHCA and spotted onto a MALDI target plate. The resulting MALDI-MS spectrum is displayed in Figure 1A. This spectrum shows an abundance of CHCA matrix related peaks in the m/z range <500, the spectrum also shows the sodium adduct ($[M+Na]^+$) of fluticasone propionate at m/z 523.2 to be slightly more intense than the protonated molecule at m/z 501.2. This may be due to increased stability of the sodiated species [16].

3.2. In-solution derivatization using DNPH

The MALDI-MS spectrum obtained for fluticasone propionate following derivatization with DNPH and using DNPH alone as matrix is shown in Figure 1B. This spectrum shows species identified as arising from the radical cation; ($[M]^+$) at m/z 500.1, the sodium adduct ($[M+Na]^+$) at m/z 523.1 (the most abundant ion), the potassium adduct ($[M+K]^+$) at m/z 539.1 and the protonated hydrazone derivative ($[M_D+H]^+$) of fluticasone propionate at m/z 681.1. The spectrum also shows the presence of the protonated reactive matrix ($[M+H]^+$) at m/z 199.0. The signals observed for fluticasone propionate are approximately ten times as intense following derivatization with DNPH compared to the "corresponding" signals in Figure 1A. The assignment of m/z 500.1 as the radical cation [$M^{+\cdot}$] for fluticasone propionate was previously explained as arising due to the

electron withdrawing effects of the attached nitro groups [5]. In order to test that this assignment was correct and that m/z 500.1 did not arise simply from in source fragmentation of the protonated hydrazone derivative an MS/MS experiment was carried out. Figure 1C shows a product ion scan of m/z 681.3, as can be seen there is no evidence of m/z 500.1 in this spectrum suggesting that this is not a preferred pathway for the dissociation and hence fragmentation of the hydrazone derivative and supports the identification of m/z 500.1 as arising from the radical cation.

During the analysis of DNPH derivatized budesonide and dexamethasone, the presence of a strong sodium adduct ion signal $[M+Na]^+$ in addition to ions corresponding to the radical cation and protonated hydrazone derivative were observed. This has been previously reported [5]. Additionally the intensity of the sodium adduct was reported to be higher than that of the radical cation, this was also observed in the work reported here, as shown in Figure 1B. The ratio between the sodium adduct and the radical cation observed in Figure 1B, could possibly be due to laser desorption ionization of unreacted fluticasone propionate due to the use of higher laser energies.

In contrast to previous work where negative ion mode is used for the analysis of DNPH derivatized aldehydes and ketones [17], here the analysis of DNPH derivatized corticosteroids was found to give a better response in positive ion mode. To confirm this DNPH dertivatized fluticasone propionate was analysed in negative ion mode (data not shown), the spectrum did not show the presence of a deprotonated hydrazine derivative or related peaks. This was also observed in previous work [5]. Also of note, even though the analyzed corticosteroids contain two or more carbonyl functionalities, due to the steric accessibility only mono substituted hydrazone derivatives ($[M_D+H]^+$) were observed.

One of the issues with the use of DNPH as a matrix is the crystallisation pattern, DNPH crystallises in long needles that does not properly cover the surface of the sample. It has previously been shown that a mixture of DNPH and CHCA in equal proportions maintains the reactive function of DNPH, whilst the CHCA promotes an even crystal coverage [7]. In order to determine if this could further improve the sensitivity and detection of the derivatized steroids a 1:1 mixture of CHCA and DNPH was employed. The MALDI-MS spectrum of

fluticasone propionate following derivatization with DNPH and subsequent analysis with CHCA as matrix is shown in Figure 1D. This MALDI-MS spectrum shows the radical cation ($[M]^+$) at m/z 500.1 (the most abundant ion), the sodium adduct ($[M+Na]^+$) at m/z 523.1, the potassium adduct ($[M+K]^+$) at m/z 539.1 and the protonated hydrazone derivative ($[M_D+H]^+$) of fluticasone propionate at m/z 681.1. The spectrum also shows a high abundance of CHCA related adduct ions in the m/z range <500. The addition of CHCA to the DNPH has changed the ratio between the radical cation ($[M]^+$) at m/z 500.1 and sodium adduct ($[M+Na]^+$) at m/z 523.1 of fluticasone propionate. Also the spectrum shows the addition of CHCA as MALDI matrix appears to improve the intensity of the radical cation and the protonated hydrazone derivative ion by approximately a factor of 2 compared to the use of DNPH alone.

3.3. In-solution derivatization using DMNTH

The MALDI-MS spectrum obtained for fluticasone propionate following derivatization with DMNTH is shown in Figure 2A. The spectrum shows an abundant ion at m/z 793.3 which corresponds to the protonated hydrazone derivative ($[M_D+H]^+$) of fluticasone propionate. Also present is the protonated un-reacted DMNTH ($[M+H]^+$) ion at m/z 311.2.

Derivatizing fluticasone proprionate DMNTH and subsequent analysis with CHCA as matrix gave a more complex spectrum (Figure 2B). This spectrum shows the protonated hydrazone derivative ($[M_D+H]^+$) at m/z 793.3 of fluticasone propionate. The spectrum also shows the presence of protonated un-reacted DMNTH ($[M+H]^+$) at m/z 311.1 which is the most abundant peak. The use of CHCA improves the intensity of the protonated hydrazone derivative signal at m/z 793.3 by approximately a factor of 10.

3.4. On-tissue derivatization using DNPH

Following the successful application of DNPH and DMNTH for the in-solution derivatization of the range of glucocorticoids, the next step was to apply these reagents for on-tissue derivatization experiments. The MALDI-MS images shown in Figure 3 demonstrate the application of the reactive matrix DNPH for the derivatization of fluticasone propionate on a control rat lung tissue section.

The MALDI-MS images displayed in Figure 3 shows the distribution of the radical cation ($[M]^{+\cdot}$) m/z 500.2 and the protonated hydrazone derivative ($[M_D+H]^+$) m/z 681.2 of fluticasone propionate within the spotted areas of the lung tissue. However, the lack of linearity of the response with respect to concentration needs to be investigated further. The cause may be related to the compound spiking methods and/ or matrix application.

3.5. On-tissue derivatization using DMNTH

The MALDI-MS images displayed in Figure 4 show the application of the reactive matrix DMNTH for the derivatization of fluticasone propionate on a control rat lung tissue section without incubation prior to analysis. These images show the distribution of the protonated hydrazone derivative ($[M_D+H]^+$) at m/z 793.2, the distribution of the reactive matrix related peak at m/z 589.2 and the protonated hydrazone derivative ($[M_D+H]^+$) at m/z 793.2 following normalisation with the reactive matrix peak at m/z 589.2. The distribution of the protonated hydrazone derivative ($[M_D+H]^+$) at m/z 793.2 is within the spotted areas of the tissue and the normalised image shows that it is detectable down to 500 ng/µL even without an incubation period.

Figure 5 shows data obtained from the same experiment but with the addition of a 48 hour, 37 °C incubation period. The rationale for the inclusion of the incubation period was to attempt to increase the yield of derivatized product, by placing the sample in a humid environment the derivatisation reagent is kept in a liquid state on the surface of the spotted tissue section thereby maintaining the reaction between the corticosteroid and the derivatisation reagent. The increased reaction time is required due to the steric accessibility of the carbonyl functionalities. The MALDI-MS images shown in Figure 5 shows the distribution of the protonated hydrazone derivative ($[M_D+H]^+$) at m/z 793.2, the distribution of the reactive matrix related peak at m/z 589.2 and the protonated hydrazone derivative ($[M_D+H]^+$) at m/z 793.2 following normalisation with the reactive matrix peak at m/z 589.2. The distribution of the protonated hydrazone derivative ($[M_D+H]^+$) at m/z 793.2 is within the spotted areas of the tissue and the normalised image shows that it is detectable down to 50 ng/µL following a 48 hour incubation at 37 °C. Hence incubation of DMNTH coated samples at 37°C for 48 hours appeared to improve the detection of the protonated hydrazone derivative by a factor of 10. No visible evidence of tissue degradation was observed for this duration and temperature.

Some preliminary data obtained from the application of this methodology to other corticosteroids is shown in Figure 6. Here derivatization of fluticasone propionate, beclomethasone dipropionate, budesonide and dexamethasone on a control rat lung tissue section has been carried out. As previously described the DMNTH coated tissue section was incubated at 37 °C for 48 hours (prior to analysis). These data show the distribution of the protonated hydrazone derivatives ($[M_D+H]^+$) of the four steroids on-tissue for; fluticasone propionate at m/z 793.2, for beclomethasone dipropionate at m/z 813.2, for budesonide at m/z 723.2 and for dexamethasone at m/z 685.2. As can be seen signals for each corticosteroid as its hydrazone derivative were observed within the respective spotted areas of the tissue. These data indicate that DMNTH is capable of derivatizing a number of corticosteroids on-tissue and that this is a methodology worthy of further study.

Conclusions

The use of hydrazine based reagents has successfully been employed for the derivatization of glucocorticoids to form their respective hydrazones (both in-solution and on-tissue) and thus improve their mass spectral ionisation efficiency and detection. The application of DNPH to the on-tissue experiments was also successful, the presence of the radical cation ($[M]^{+\cdot}$) and the protonated hydrazone derivative ($[M_D+H]^+$) of fluticasone propionate was observed in the spotted areas of the tissue sections. However further studies are required for the application of DNPH to on-tissue experiments to explain the apparent lack of linearity for the response with respect to concentration spotted onto the tissue section.

The “tailor-made” reactive matrix DMNTH has successfully been applied to the in-solution derivatization of glucocorticoids. The application of DMNTH to on-tissue experiments has also been successful, it has been shown that analysis of samples immediately after reactive matrix application results in the detection of the protonated hydrazone derivative ($[M_D+H]^+$) of fluticasone propionate at a concentration of 500 ng/µL. It has been shown that a prolonged reaction (~48 hours) improves the detection of the protonated hydrazone derivative. It was found that following incubation in a humid environment (37°C) results in the detection of the protonated hydrazone derivative ($[M_D+H]^+$) of fluticasone propionate at a concentration of 50 ng/µL.

Further improvements in sensitivity and limits of detection were observed when CHCA was added to the reactive matrices DNPH or DMNTH.

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Figure Legends

Figure 1: A) MALDI-MS spectrum showing fluticasone propionate (500 ng/µL) mixed with the matrix CHCA. B) MALDI-MS spectrum showing fluticasone propionate (500 ng/µL) following 1 hour reaction with the reactive matrix DNPH at room temperature. C) MALDI-MS/MS spectrum of m/z 681.3 the protonated hydrazone derivative ($[M_D+H]^+$) of fluticasone proprionate. D) MALDI-MS spectrum showing fluticasone propionate (500 ng/µL) following 1 hour reaction with a mixture of the reactive matrix DNPH and CHCA at room temperature.

Figure 2: A) MALDI-MS spectrum showing fluticasone priopionate (500 ng/µL) following 48 hour reaction with the reactive matrix DMNTH at room temperature.. B) MALDI-MS spectrum showing fluticasone priopionate (500 ng/µL) following 48 hour reaction with a mixture of the reactive matrix DMNTH and CHCA at room temperature.

Figure 3: Optical images showing the spotted tissue section A) before and B) after reactive matrix deposition. MALDI-MS images showing the distribution of C) the radical cation ($[M]^{+\cdot}$) of fluticasone propionate at m/z 500.2 and D) the protonated hydrazone derivative ($[M_D+H]^+$) of fluticasone propionate at m/z 681.2.

Figure 4: Optical images showing the spotted tissue section A) before and B) after reactive matrix deposition. MALDI-MS images showing the distribution of the protonated hydrazone derivative of fluticasone propionate at m/z 793.2 C) before and D) after normalisation against the distribution of E) a reactive matrix related peak at m/z 589.2.

Figure 5: Optical images showing the spotted tissue section A) before and B) after reactive matrix deposition. MALDI-MS images showing the distribution of the protonated hydrazone derivative of fluticasone propionate at m/z 793.2 C) before and D) after normalisation against the distribution of E) a reactive matrix related peak at m/z 589.2.

Figure 6: Optical images showing the spotted tissue section A) before and B) after reactive matrix deposition. MALDI-MS images showing the distribution of the protonated hydrazone derivatives of C) fluticasone

propionate at m/z 793.2, D) beclomethasone dipropionate at m/z 813.2, E) budesonide at m/z 723.2, F) dexamethasone at m/z 685.2 normalised against the distribution of G) a reactive matrix related peak at m/z 589.2.

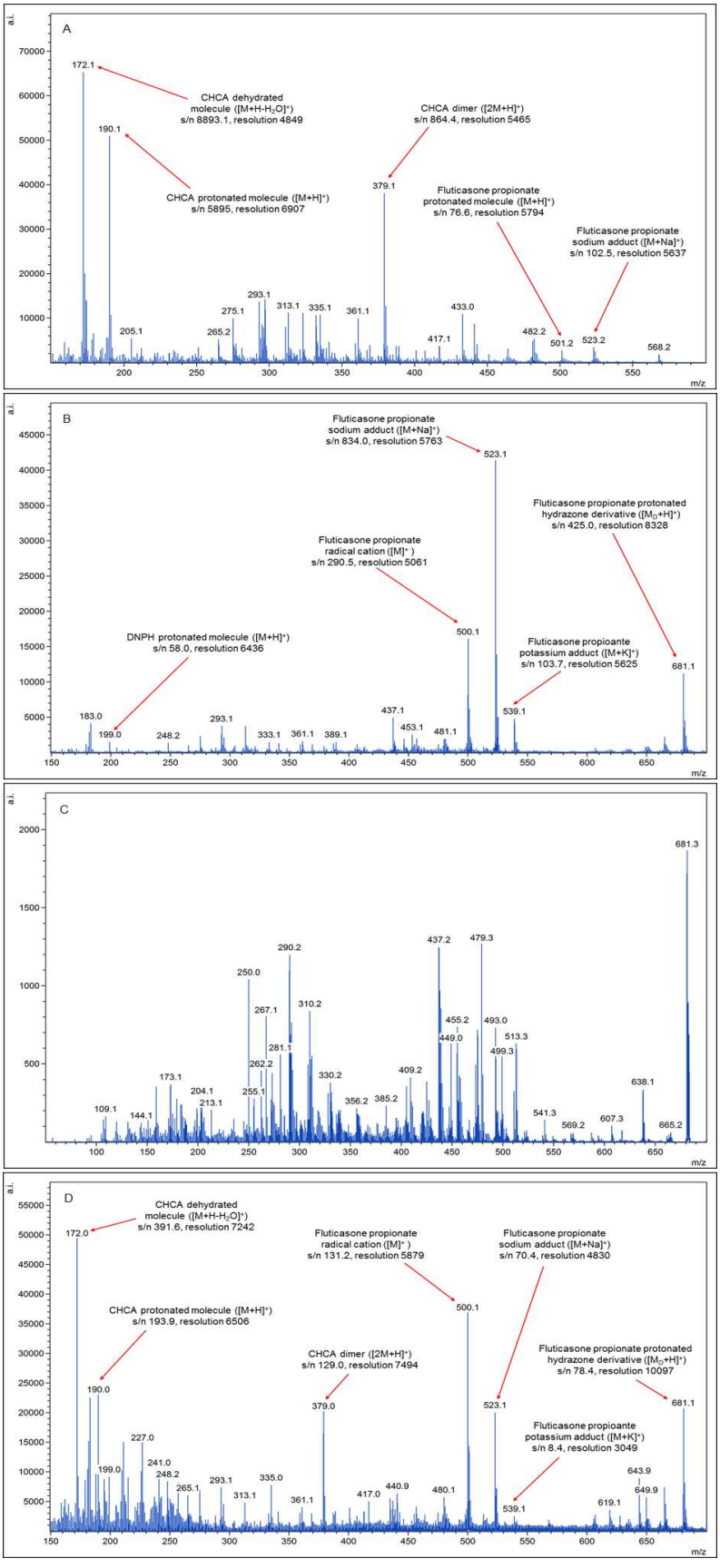
A
CHCA dehydrated molecule ([M+H-H2O]+) s/n 8893.1, resolution 4849
CHCA protonated molecule ([M+H]+) s/n 5895, resolution 6907
CHCA dimer ([2M+H]+) s/n 864.4, resolution 5465
Fluticasone propionate protonated molecule ([M+H]+) s/n 76.6, resolution 5794
Fluticasone propionate sodium adduct ([M+Na]+) s/n 102.5, resolution 5637
B
Fluticasone propionate sodium adduct ([M+Na]+) s/n 834.0, resolution 5763
Fluticasone propionate protonated hydrazone derivative ([M2+H]+) s/n 425.0, resolution 8328
Fluticasone propionate radical cation ([M]+) s/n 290.5, resolution 5061
DNPH protonated molecule ([M+H]+) s/n 58.0, resolution 6436
Fluticasone propioante potassium adduct ([M+K]+) s/n 103.7, resolution 5625
C
D
CHCA dehydrated molecule ([M+H-H2O]+) s/n 391.6, resolution 7242
Fluticasone propionate radical cation ([M]+) s/n 131.2, resolution 5879
Fluticasone propionate sodium adduct ([M+Na]+) s/n 70.4, resolution 4830
CHCA protonated molecule ([M+H]+) s/n 193.9, resolution 6506
CHCA dimer ([2M+H]+) s/n 129.0, resolution 7494
Fluticasone propionate protonated hydrazone derivative ([M2+H]+) s/n 78.4, resolution 10097
Fluticasone propioante potassium adduct ([M+K]+) s/n 8.4, resolution 3049
a.i.
m/z

Figure 1

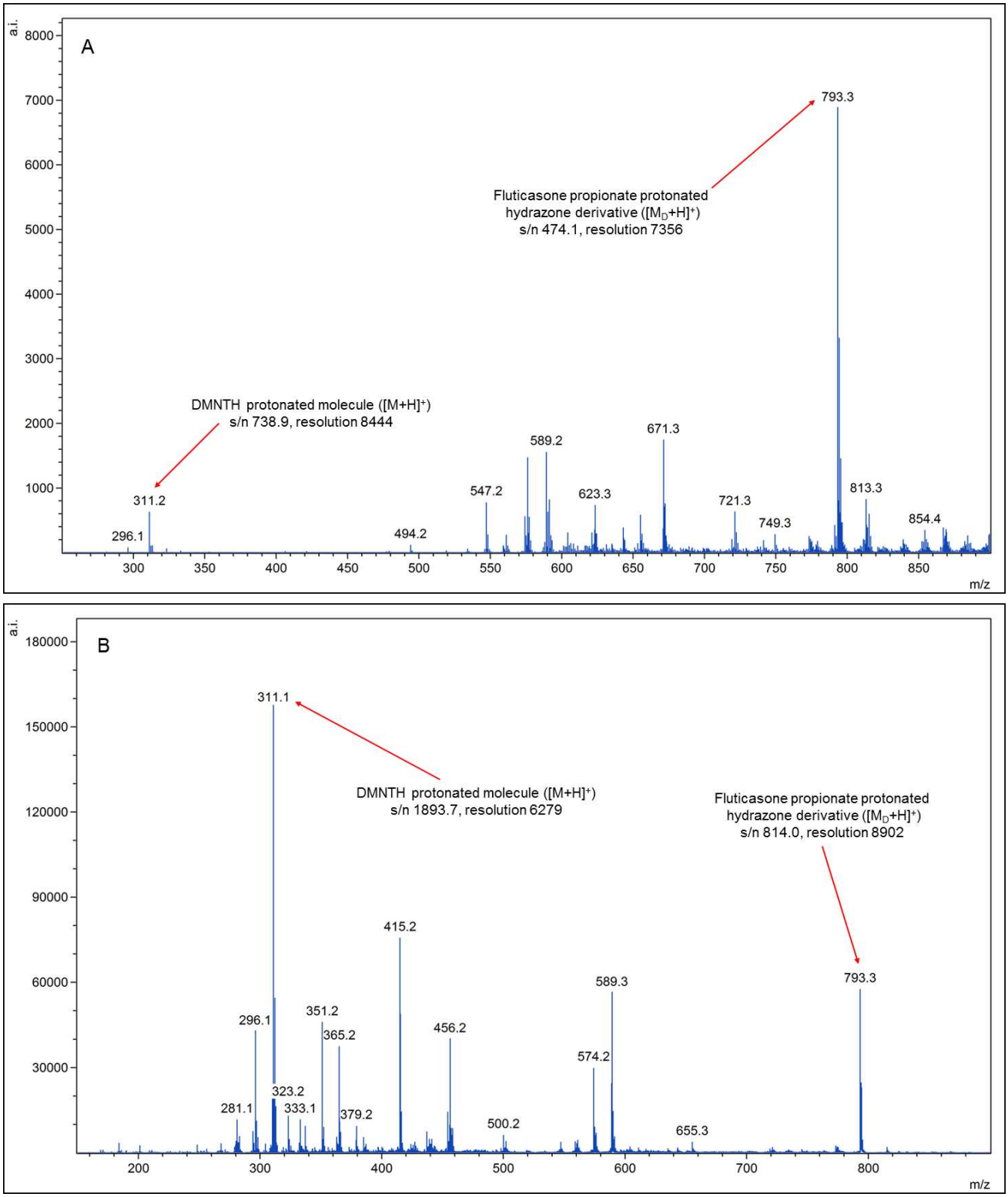
A
a.i.
8000
7000
6000
5000
4000
3000
2000
1000
793.3
Fluticasone propionate protonated
hydrazone derivative ([M$_D$+H]$^+$)
s/n 474.1, resolution 7356
DMNTH protonated molecule ([M+H]$^+$)
s/n 738.9, resolution 8444
671.3
589.2
547.2
623.3
311.2
721.3
813.3
749.3
854.4
296.1
494.2
300
350
400
450
500
550
600
650
700
750
800
850
m/z
B
a.i.
180000
150000
120000
90000
60000
30000
311.1
DMNTH protonated molecule ([M+H]$^+$)
s/n 1893.7, resolution 6279
Fluticasone propionate protonated
hydrazone derivative ([M$_D$+H]$^+$)
s/n 814.0, resolution 8902
415.2
589.3
793.3
296.1
351.2
365.2
456.2
574.2
323.2
281.1
333.1
379.2
500.2
655.3
200
300
400
500
600
700
800
m/z

Figure 2

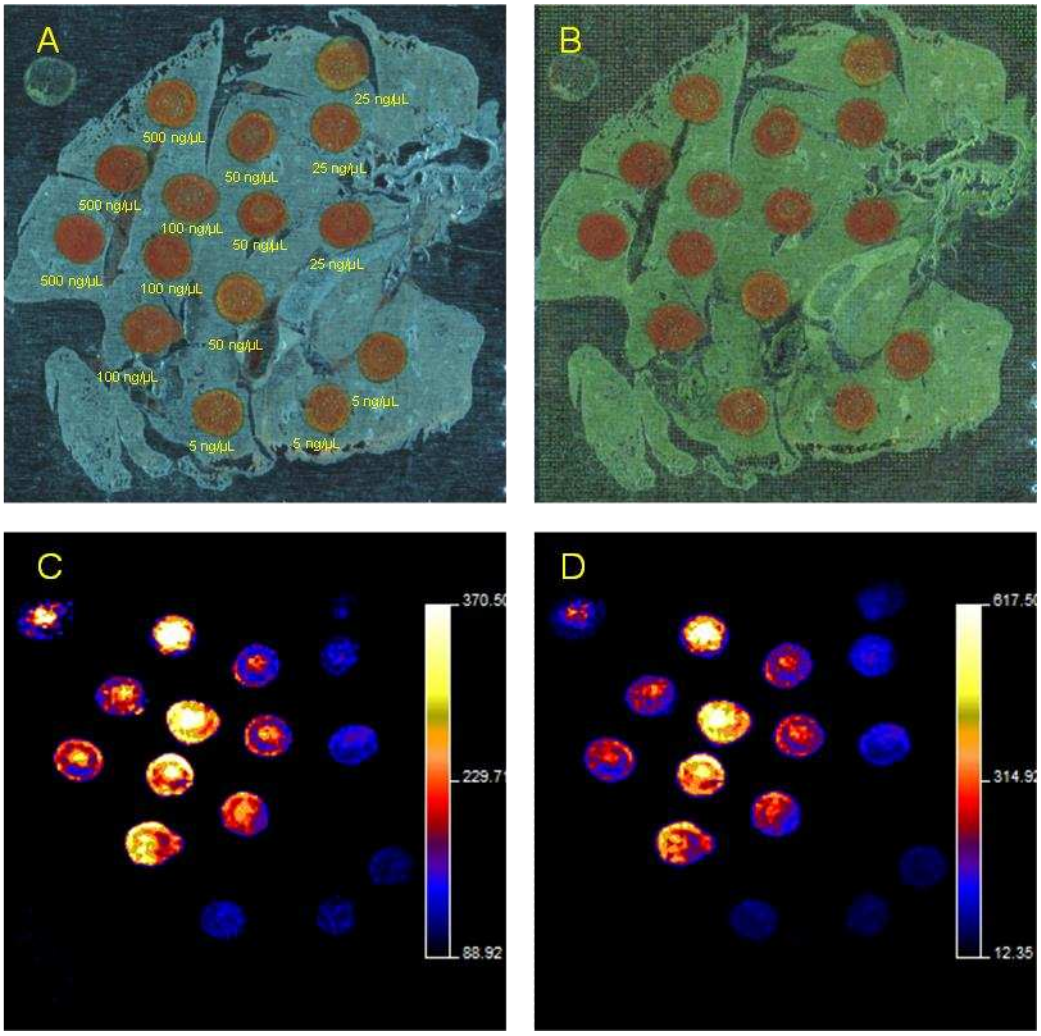
A
25 ng/µL
500 ng/µL
50 ng/µL
25 ng/µL
500 ng/µL
100 ng/µL
50 ng/µL
25 ng/µL
500 ng/µL
100 ng/µL
50 ng/µL
100 ng/µL
5 ng/µL
5 ng/µL
5 ng/µL
B
C
370.5
229.7
88.92
D
617.5
314.9
12.35

Figure 3

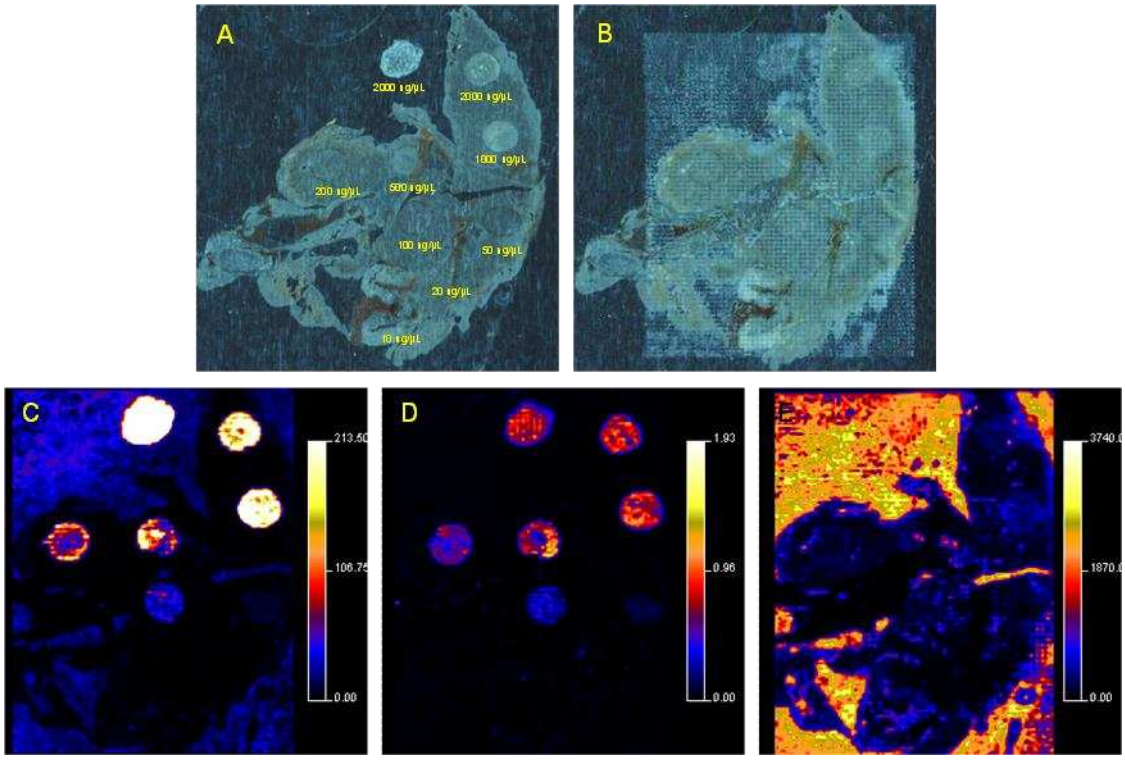
A
2000 ng/μL
2000 ng/μL
1000 ng/μL
200 ng/μL
500 ng/μL
100 ng/μL
50 ng/μL
20 ng/μL
B
C
213.50
106.75
0.00
D
1.93
0.96
0.00
3740.0
1870.0
0.00

Figure 4

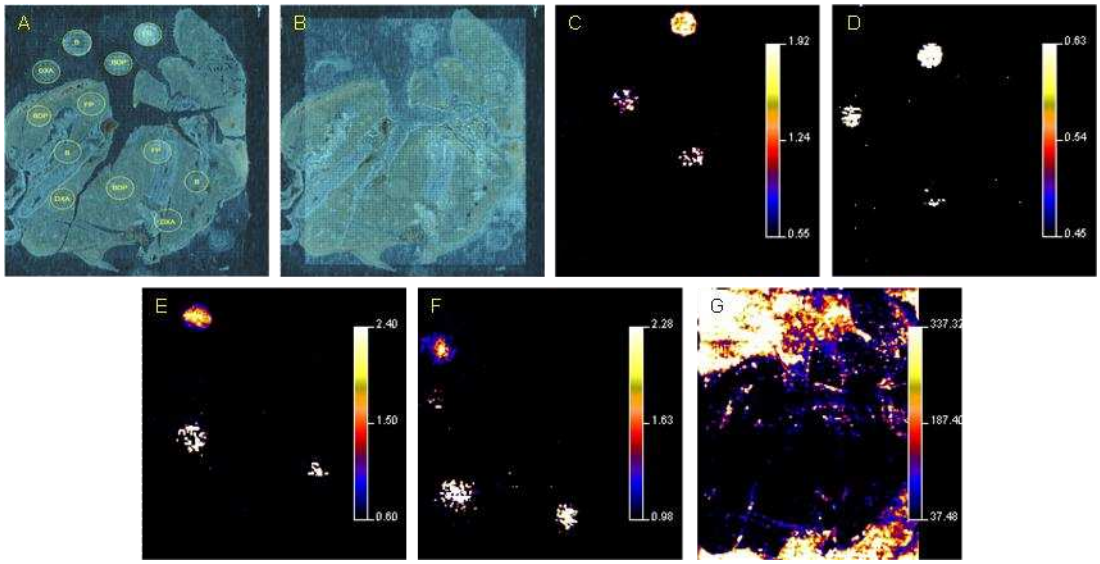
A
B
C
1.92
1.24
0.55
D
0.63
0.54
0.45
E
2.40
1.50
0.60
F
2.28
1.63
0.98
G
337.32
187.40
37.48

Figure 5